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# Calcium in Renal Cells. Modulation of Calcium-Dependent Activation of Phospholipase A<sub>2</sub>

by Joseph V. Bonventre\*

Calcium has been implicated as a regulatory factor in many physiological and pathophysiological processes in the renal cell. Under physiological conditions, the cytosolic free calcium concentration is maintained at approximately 100 nM. Most of the releasable cell Ca<sup>2+</sup> resides in the nonmitochondrial compartments. In addition to the plasma membrane Ca<sup>2+</sup> transport processes, there is a high-affinity, low-capacity buffering capability of nonmitochondrial organelles and a lower-affinity high-capacity mitochondrial Ca<sup>2+</sup> buffering capability.

A critical enzymatic effector of Ca<sup>2+</sup> action in the cell is phospholipase A<sub>2</sub>. By using digitonin-permeabilized renal mesangial cells, the [Ca<sup>2+</sup>] dependency of phospholipase A<sub>2</sub> was characterized. The [Ca<sup>2+</sup>] sensitivity was insufficient to explain the phospholipase A<sub>2</sub> activation observed with vasopressin. In both intact cells, as well as permeabilized cells, it was found that protein kinase C activation markedly enhanced the Ca<sup>2+</sup> calmodulin-dependent activation of phospholipase A<sub>2</sub>.

In response to platelet-derived growth factor, it was found that arachidonic acid release preceded phospholipase C activation. This suggests that other effectors besides Ca<sup>2+</sup> and protein kinase C may also be important for phospholipase A<sub>2</sub> activation.

In an experimental model designed to mimic postischemic reperfusion damage to renal mitochondria, it was demonstrated that reactive oxygen species act synergistically with Ca<sup>2+</sup> to activate mitochondrial phospholipase A<sub>2</sub>, which mediates damage to site I of the electron transport chain, the F<sub>1</sub>F<sub>0</sub> ATPase, and the adenine nucleotide translocase.

In conclusion, an adequate understanding of the physiological and pathophysiological roles of intracellular Ca<sup>2+</sup> relies, not only on the measurement of Ca<sup>2+</sup> concentration and the characterization of "Ca<sup>2+</sup>-dependent" processes, but an appreciation of the complex synergistic interactions between Ca<sup>2+</sup> and other mediators of cellular activation and toxicity.

## Cytosolic Ca<sup>2+</sup> Concentration and Ca<sup>2+</sup> Homeostasis

Calcium has been implicated in a number of physiological and pathophysiological processes in the renal cell. By using three different techniques for measuring cytosolic free Ca<sup>2+</sup> concentration ([Ca<sup>2+</sup>]<sub>f</sub>), Quin 2, Fura-2 and the null point titration method, we found [Ca<sup>2+</sup>]<sub>f</sub> to be approximately 100 nM in cultured renal epithelial cells (LLC-PK<sub>1</sub>) and cultured mesangial cells (1,2).

Regulation of [Ca<sup>2+</sup>]<sub>f</sub> at these low levels depends on the functioning of membrane transport systems. At the plasma membrane the Ca<sup>2+</sup> ATPase and, likely, a Na<sup>+</sup>/Ca<sup>2+</sup> exchanger (1) function to transport Ca<sup>2+</sup> out of the cell. In the LLC-PK<sub>1</sub> we have found that, under basal conditions, only a small amount (approximately 10%) of releasable Ca<sup>2+</sup> resides in the mitochondrial compartment. In the presence of physiological levels

of Mg<sup>2+</sup> and ATP, small amounts of Ca<sup>2+</sup> added to digitonin-permeabilized cells are taken up via a vanadate-inhibitable, ruthenium red-insensitive process into a nonmitochondrial compartment (3). This compartment buffers Ca<sup>2+</sup> such that [Ca<sup>2+</sup>]<sub>f</sub> is maintained in the 50 to 300 nM range. Thus, under physiological conditions, most of the cell Ca<sup>2+</sup> stored in membrane-bound releasable form is located in a nonmitochondrial compartment that may be the endoplasmic reticulum, a subcompartment of the endoplasmic reticulum, or other organelles (4). The Ca<sup>2+</sup> transport characteristics of this compartment are likely to be important for the maintenance of cytosolic Ca<sup>2+</sup> concentrations close to basal levels, in spite of minor fluctuations in plasma membrane transport or permeability characteristics. With the continued addition of Ca<sup>2+</sup> to permeabilized cells, this compartment is saturated with Ca<sup>2+</sup>, and the additional Ca<sup>2+</sup> is buffered by the mitochondria, a high-capacity Ca<sup>2+</sup> buffering compartment. While mitochondria can take up very large amounts of Ca<sup>2+</sup>, the resultant free extramitochondrial Ca<sup>2+</sup> concentration is in the 600 to

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1500 nM range. Thus, when large amounts of  $\text{Ca}^{2+}$  are presented to the cell, as when the cell is exposed to a toxic or ischemic influence, the nonmitochondrial stores are rapidly saturated, and the mitochondria assume the primary role of  $\text{Ca}^{2+}$  buffer (3).

## Physiological and Pathophysiological Roles of Phospholipase $\text{A}_2$

One of the effector enzymes that serves as a critical transducer of  $\text{Ca}^{2+}$  action in the cell is phospholipase  $\text{A}_2$ . This enzyme cleaves the fatty acid from the sn-2 position of phospholipids. Since many cellular phospholipids are enriched in arachidonic acid in the sn-2 position, phospholipase  $\text{A}_2$  activation results in enhanced levels of free arachidonic acid, which is the precursor for an entire family of metabolically active substances: prostaglandins, leukotrienes, Hete's, epoxides, and thromboxanes.

It is believed by most, but not all, investigators that the production of free arachidonic acid is rate limiting for synthesis of these compounds, thus placing phospholipase  $\text{A}_2$  at the critical regulatory step. With the exception of phospholipase  $\text{A}_2$  of lysosomal origin, most of the phospholipase  $\text{A}_2$  enzymes characterized to this point require  $\text{Ca}^{2+}$  for activation (5).

Free arachidonic acid and its products may serve as intracellular messengers (6) and may activate other phospholipases involved in the signal cascade modulating the cellular responses to activating stimuli. In addition to these effects on signal transduction, many of the results of phospholipase  $\text{A}_2$  activation may be manifested on other cells in the tissue of origin or other tissues in the organism since many of the arachidonic acid cascade products are secreted (7,8).

In addition to the physiological regulatory effects of phospholipase  $\text{A}_2$  activation products, there are many potential adverse consequences to the cell and organism (9). For example, prostaglandins, leukotrienes, and thromboxanes are mediators of inflammation (10). Also, the free fatty acids (11-15) and lysophospholipids (16) that result from phospholipase  $\text{A}_2$ -induced phospholipid hydrolysis are very toxic to the cell. The mitochondria are important targets of phospholipase  $\text{A}_2$  action, resulting in adverse consequences to the cell. Activation of mitochondrial phospholipase  $\text{A}_2$  results in hydrolysis of cardiolipin (diphosphatidylglycerol), a lipid that is a critical component of the catalytic subunits of the electron transport chain (complexes I, III, and cytochrome oxidase) (17), ATP synthase (18,19), and adenine nucleotide translocase (20). Cone-shaped lipids, such as cardiolipin, may increase the susceptibility of other membrane phospholipids to phospholipase  $\text{A}_2$  action by decreasing the packing of polar head groups (21).

Therefore, because of the implications for understanding the physiological response of cells to activating stimuli and the need to understand the events triggering cell and tissue injury, it is important to

understand the regulation of phospholipase  $\text{A}_2$  and the role played by  $\text{Ca}^{2+}$  in its activation.

## $\text{Ca}^{2+}$ -Dependent Activation of Phospholipase $\text{A}_2$

A standard model for cell activation by an agonist, such as vasopressin resulting in phospholipase  $\text{A}_2$  activation, can be described as follows: Hormone-receptor interaction results in the activation of phospholipase C. This process is mediated by a guanine nucleotide-binding protein in many cells, including the mesangial cell (22). The action of phospholipase C on polyphosphoinositides results in the generation of inositol trisphosphate ( $\text{IP}_3$ ) and diacylglycerol.  $\text{IP}_3$  acts upon nonmitochondrial  $\text{Ca}^{2+}$  storage sites to release  $\text{Ca}^{2+}$ , increasing  $[\text{Ca}^{2+}]_i$ . The increase in  $[\text{Ca}^{2+}]_i$  results in the activation of phospholipase  $\text{A}_2$  and the subsequent generation of eicosanoids.

We have examined the  $\text{Ca}^{2+}$ -dependent regulation of phospholipase  $\text{A}_2$  in the glomerular mesangial cells in culture. These cells contract, produce eicosanoids, proliferate in conditions of disease, and produce matrix components that may be important in the pathophysiology of renal disease. When vasopressin activates mesangial cells,  $[\text{Ca}^{2+}]_i$  increases to levels of approximately 600 nM (2). This increase in  $[\text{Ca}^{2+}]_i$  is due primarily to its release from intracellular storage sites, since the reduction of extracellular  $[\text{Ca}^{2+}]$  with EGTA only modulates the response minimally. There is an associated 10-fold increase in prostaglandin  $\text{E}_2$  production by these cells that is not affected by changing extracellular  $[\text{Ca}^{2+}]$  over three ranges (< 100 nM, 1-10  $\mu\text{M}$ , 1.5 mM) (22). This insensitivity of prostaglandin synthesis to extracellular  $[\text{Ca}^{2+}]$  is consistent with the view that release of  $\text{Ca}^{2+}$  from intracellular stores, rather than entry of  $\text{Ca}^{2+}$  across the plasma membrane, may be responsible for the prostaglandin  $\text{E}_2$  production.

The level of the  $\text{Ca}^{2+}$  concentration necessary to stimulate phospholipase  $\text{A}_2$  in many assay systems, however, is significantly higher than that achieved with hormone activation (22,23). Likewise, under conditions of ischemic and toxic injury,  $[\text{Ca}^{2+}]_i$  increases to levels lower than those predicted to be necessary to activate the enzyme *in vitro*, although damage has occurred to the cell that has been proposed to be phospholipase  $\text{A}_2$  related (24,25).

It is possible that these apparent inconsistencies between the  $\text{Ca}^{2+}$  concentrations necessary to activate the enzyme in cell-free systems and the levels achieved in the intact cell may be due to the absence of important cofactors or the enhanced expression of endogenous inhibitor activity in the assay system *in vitro* (26). Alternatively, other modulatory factors that are present in the intact cell upon activation but absent in the cell-free system may account for the differences.

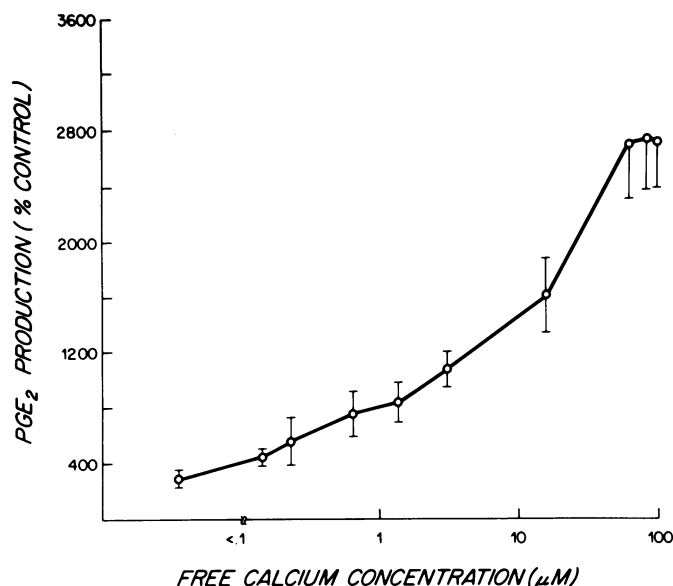


FIGURE 1. Prostaglandin  $\text{E}_2$  ( $\text{PGE}_2$ ) production as a function of free  $\text{Ca}^{2+}$  concentration in digitonin-permeabilized renal mesangial cells. Media  $\text{PGE}_2$  content was determined by radioimmunoassay 10 min after addition of digitonin. Each data point represents the mean  $\pm$  SE of 4 to 10 experiments. Modified from Bonventre and Swidler (22).

In order to address this issue, we examined the  $\text{Ca}^{2+}$  dependency of phospholipase  $\text{A}_2$  activity under experimental conditions designed to maintain the enzyme in its normal configuration in the cell.  $\text{PGE}_2$  synthesis was measured as a function of cytosolic  $[\text{Ca}^{2+}]$  in cells rendered permeable with digitonin.  $[\text{Ca}^{2+}]$  was clamped secondary to the large amount of incubation media relative to cell mass and the free communication between the cytosolic compartment and bath.  $[\text{Ca}^{2+}]$  was measured with a  $\text{Ca}^{2+}$  electrode (2). When  $[\text{Ca}^{2+}]$  varied over the range from < 100 nM to 100  $\mu\text{M}$ , there was a progressive increase in  $\text{PGE}_2$  production (Fig. 1). The increase was modest, but nevertheless apparent over the physiological range (100 nM–2  $\mu\text{M}$ ). Calmodulin inhibitors (22,27) decreased  $\text{PGE}_2$  synthesis at all concentrations of  $[\text{Ca}^{2+}]$ . The addition of exogenous calmodulin had no effect on  $\text{PGE}_2$  production at any level of  $[\text{Ca}^{2+}]$ , presumably because the cell permeabilization procedure did not result in loss of calmodulin in sufficient quantities to compromise phospholipase  $\text{A}_2$  function (22).

To establish that the increase in  $\text{PGE}_2$  production was associated with an increase in acylhydrolase activity, arachidonic acid release was measured in cells prelabeled with  $^3\text{H}$ -arachidonic acid. Release was determined as a function of  $\text{Ca}^{2+}$  with  $[\text{Ca}^{2+}]$  fixed at three different levels (< 100 nM, 1–10  $\mu\text{M}$ , and 500  $\mu\text{M}$ ) in digitonin-permeabilized cells. As depicted in Table 1, free arachidonic acid increased as a function of  $\text{Ca}^{2+}$  concentration in a manner that paralleled the  $\text{PGE}_2$  production. In addition,  $^3\text{H}$ -diglyceride levels were increased with increasing  $[\text{Ca}^{2+}]$ . This latter finding of increases in diacylglycerol with increasing

Table 1. Free  $^3\text{H}$ -arachidonic acid and  $^3\text{H}$ -diglyceride in digitonin-treated cells as a function of  $[\text{Ca}^{2+}]$ .<sup>a</sup>

$\text{Ca}^{2+}$ concentration	% Total radioactivity	
	$^3\text{H}$ -Arachidonic acid	$^3\text{H}$ -Diglyceride
< 100 nM	$1.7 \pm 0.3$	$0.7 \pm 0.1$
1–10 $\mu\text{M}$	$5.4 \pm 1.9$	$1.6 \pm 0.4$
500 $\mu\text{M}$	$14.9 \pm 7.0$	$2.5 \pm 0.5$

<sup>a</sup>Cells were prelabeled with  $^3\text{H}$ -arachidonic acid. They were then permeabilized with digitonin in the presence of three different ranges of calcium concentration. After 10 min cells and supernatant were combined and assayed for free  $^3\text{H}$ -arachidonate and  $^3\text{H}$ -diglyceride by thin-layer chromatography after chloroform-methanol extraction (22). Results represent the mean  $\pm$  SEM of four experiments. Each value of  $^3\text{H}$ -arachidonic acid and  $^3\text{H}$ -diglyceride was statistically different from values obtained at different  $[\text{Ca}^{2+}]$  ranges ( $p < 0.05$ ) (22). Data and table from Bonventre and Swidler (22).

$\text{Ca}^{2+}$  concentration indicates that phospholipase  $\text{C}$  activity in the mesangial cell is sensitive to  $\text{Ca}^{2+}$ . W-7 (50  $\mu\text{M}$ ), a calmodulin inhibitor (28), inhibited the arachidonic acid release. Furthermore, the phospholipase  $\text{A}_2$  inhibitors, dibucaine and mepacrine, inhibited the  $\text{Ca}^{2+}$ -dependent arachidonic acid release, but they had no effect on the  $\text{Ca}^{2+}$ -dependent increases in  $^3\text{H}$ -diglyceride (22).

In summary, these observations are consistent with a  $\text{Ca}^{2+}$ , calmodulin-dependent activation of phospholipase  $\text{A}_2$ . While these data indicate that phospholipase  $\text{A}_2$  can be activated when  $[\text{Ca}^{2+}]$  is varied over the physiological range, the degree of activation is not very great. When the stimulation of  $\text{PGE}_2$  production (in response to vasopressin) is compared to that observed in permeabilized cells (in response to  $\text{Ca}^{2+}$  concentration increases to levels seen in vasopressin-stimulated intact cells), it is clear that the increase in  $[\text{Ca}^{2+}]_i$  observed with vasopressin, is insufficient to explain the  $\text{PGE}_2$  production seen with vasopressin (Fig. 2).

### Protein Kinase C Enhancement of $\text{Ca}^{2+}$ -Dependent Phospholipase $\text{A}_2$ Activity and $\text{PGE}_2$ Production

As indicated previously, when vasopressin and other agonists stimulate phospholipase  $\text{C}$ , there is a resultant increase in protein kinase  $\text{C}$  activation along with the rise in  $[\text{Ca}^{2+}]_i$ . We examined whether or not protein kinase  $\text{C}$  activation modulates the calcium dependency of phospholipase  $\text{A}_2$  stimulation.  $\text{PGE}_2$  production in permeabilized cells was compared in the presence or absence of PMA when  $[\text{Ca}^{2+}]_i$  was fixed either at basal levels (< 200 nM) or in the approximate range achieved in vasopressin-stimulated cells (between 0.3 and 2.2  $\mu\text{M}$ ). When  $\text{Ca}^{2+}$  was fixed at basal levels, there was no effect of PMA on  $\text{PGE}_2$  synthesis.

By contrast, however, when  $\text{Ca}^{2+}$  was fixed at the higher levels in order to mimic the levels of  $[\text{Ca}^{2+}]_i$

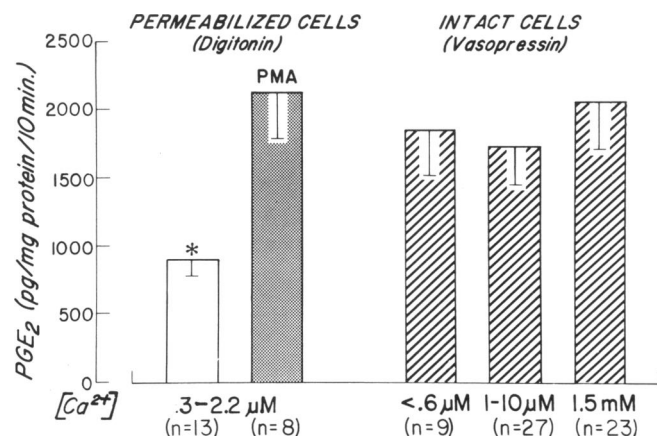


FIGURE 2. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in renal mesangial cells made permeable with digitonin in the presence of 0.3 to 2.2 μM [Ca<sup>2+</sup>] with or without simultaneous stimulation with phorbol myristate acetate (PMA, 300 nM). For comparison, the PGE<sub>2</sub> production from nonpermeabilized intact cells stimulated with vasopressin (100 nM) is presented. The intact cells were stimulated in the presence of three different ranges of extracellular [Ca<sup>2+</sup>]. Asterisk (\*) denotes ( $p < 0.001$ ) significantly different from control compared with other conditions. Modified from Bonventre and Swidler (22).

reached with vasopressin, PMA resulted in an approximate 100% increase in PGE<sub>2</sub> production over that seen with Ca<sup>2+</sup> alone (22). This increase induced by PMA resulted in a net PGE<sub>2</sub> production in the permeabilized cell that roughly equaled that in the vasopressin-stimulated intact cell (Fig. 2).

To further demonstrate that the increase in calcium concentration was necessary, albeit not sufficient to explain the enhanced levels of phospholipase A<sub>2</sub> activity, we exposed permeabilized cells to vasopressin (100 nM) in the presence of 100 μM GTPγS in order to stimulate phospholipase C. Under these conditions there was a significant increase in <sup>3</sup>H-diglyceride ( $1.55 \pm 0.14\%$  of total radioactivity to  $2.37 \pm 0.29\%$ ,  $n = 8$ ,  $p < 0.01$ ). There was, however, no increase in free arachidonic acid (22). Thus, under conditions where phospholipase C and presumably protein kinase C were activated, there was no stimulation of phospholipase A<sub>2</sub> if [Ca<sup>2+</sup>]<sub>i</sub> was maintained at low basal cellular levels.

To establish that this synergistic effect between Ca<sup>2+</sup> and PMA that was seen in permeabilized cells was also present in intact cells, we examined free arachidonic acid levels in intact cells stimulated with A23187, PMA, A23187 + PMA, or vasopressin alone for 10 min (Fig. 3). At low doses of the calcium ionophore, A23187, 0.1 μM, only a slight increase occurred in arachidonic acid release. PMA alone had no effect on arachidonic acid release. In spite of this small effect of ionophore and no effect of PMA alone, both added together resulted in a marked stimulation of arachidonic acid release, reflecting a synergistic interaction. The levels of free arachidonic acid reached with the combination of A23187 and PMA in intact cells equalled that with vasopressin (100 nM) alone. Using higher concentrations of A23187 (1 μM)

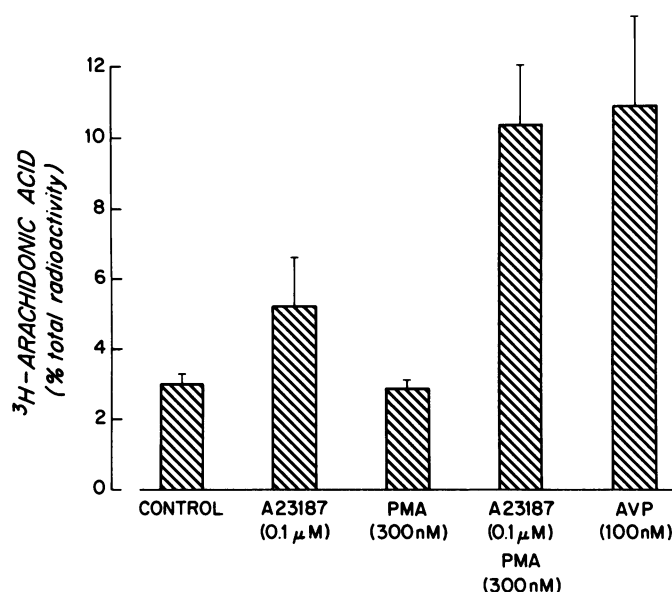


FIGURE 3. Release of free <sup>3</sup>H-arachidonic acid from intact mesangial cells stimulated for 10 min with the Ca<sup>2+</sup> ionophore, A23187; PMA alone; the combination of A23187 and PMA; or vasopressin. The data bars represent the mean  $\pm$  1 SEM of six to eight experiments. From Bonventre and Swidler (22).

and lower concentrations of PMA (10 nM), and also longer time periods for stimulation (30 min), the levels of free arachidonic acid far exceeded that of vasopressin alone (22).

To demonstrate further that this effect of PMA was due to an effect of protein kinase C and not a nonspecific effect of phorbol esters, we used the diacylglycerol analogue, 1-oleoyl 2-acetyl glycerol. This analogue also worked synergistically with A23187 to increase free arachidonic levels in intact mesangial cells (22).

We concluded from this data that agonist-induced changes in [Ca<sup>2+</sup>]<sub>i</sub> alone were not sufficient to account for the phospholipase A<sub>2</sub> activation observed with vasopressin. Our data indicate that the stimulation of protein kinase C enhances the activation of phospholipase A<sub>2</sub>. A protein kinase C-dependent phosphorylation process may, therefore, be important in enhancement of acylhydrolase activity in the mesangial cell. It is possible that protein kinase C phosphorylates phospholipase A<sub>2</sub> itself or proteins with phospholipase A<sub>2</sub> modulatory capability (29). It has been proposed that a family of proteins, referred to as lipocortins, may exert a chronic inhibitory influence on phospholipase A<sub>2</sub> that is released when the compounds are phosphorylated. In collaboration with Dr. Blake Pepinsky we have identified the presence of lipocortin I and lipocortin II in mesangial cells (22); however, there remains considerable controversy as to whether these proteins represent endogenous functionally important regulated inhibitors of phospholipase A<sub>2</sub> (30). Another possible explanation for protein kinase C-enhanced phospholipase A<sub>2</sub> activity is that protein kinase C may phosphorylate a

GTP-binding (G) protein, which interacts with phospholipase  $\text{A}_2$  to enhance hydrolase activity when the latter is stimulated by  $\text{Ca}^{2+}$ . G-proteins have been reported to modulate phospholipase  $\text{A}_2$  activity in rod outer segments of bovine retina (31) and FRTL-5 rat thyroid cells (32). Phospholipase  $\text{A}_2$  in RAW 264.7 macrophages is enhanced by both cholera toxin and pertussis toxin, suggesting stimulatory and inhibitory G-protein involvement in the process (33). Finally, Schlondorff et al. (34) and Pfeilschifter and Bauer (35) found that pertussis toxin modulated agonist-induced  $\text{PGE}_2$  synthesis. Our data, indicating no activation of phospholipase  $\text{A}_2$  with  $\text{GTP}\gamma\text{S}$  under conditions of very low cytosolic calcium concentrations, do not rule out a potential role for a GTP-binding protein in phospholipase  $\text{A}_2$  activation when cytosolic calcium levels are increased. Protein kinase C may also activate a  $\text{Na}^+/\text{H}^+$  exchange process (36), which we have identified to be present in the mesangial cell (37). This in turn may activate phospholipase  $\text{A}_2$  by a phospholipase C-independent mechanism, as has been reported in the platelet (38). This would not however explain the PMA effect in the permeabilized cell where the pH was clamped.

### Phospholipase C-Independent Activation of Phospholipase $\text{A}_2$

Many agonists that increase phospholipase  $\text{A}_2$  activity in cells increase phospholipase C activity promptly upon interaction with their receptors, resulting in an inability to distinguish a phospholipase C-dependent activation of phospholipase  $\text{A}_2$  mediated by  $\text{IP}_3$  and an increase in  $[\text{Ca}^{2+}]_i$  and protein kinase C activity from a potential phospholipase C-independent mechanism of phospholipase  $\text{A}_2$  activation. We were able to dissociate the two effects for the first time in a nucleated intact cell preparation by examining phospholipase C and phospholipase  $\text{A}_2$  activation patterns in response to platelet-derived growth factor (PDGF) (39). PDGF increases  $[\text{Ca}^{2+}]_i$  in mesangial cells. However, in contrast to vasopressin and platelet-activating factor that increased  $\text{Ca}^{2+}$  to peak levels in less than 10 sec of agonist stimulation, PDGF increases  $[\text{Ca}^{2+}]_i$  levels much more slowly, with the peak response occurring at 1 min. This slow  $[\text{Ca}^{2+}]_i$  activation pattern is seen at a number of different concentrations of PDGF, ranging from 0.005 units/mL to 5 units/mL. One possible explanation for this delay in the peak  $\text{Ca}^{2+}$  response is that PDGF-induced stimulation of phospholipase C may also be delayed. As indicated in Figure 4, when either inositol phosphate or diacylglycerol levels were determined at 15 sec after PDGF stimulation, there was no significant increase in phospholipase C activation. On the other hand, by 2 min there was an approximately 100% increase in the diacylglycerol and inositol phosphate levels. By contrast, free arachidonic levels increased by more than 100%

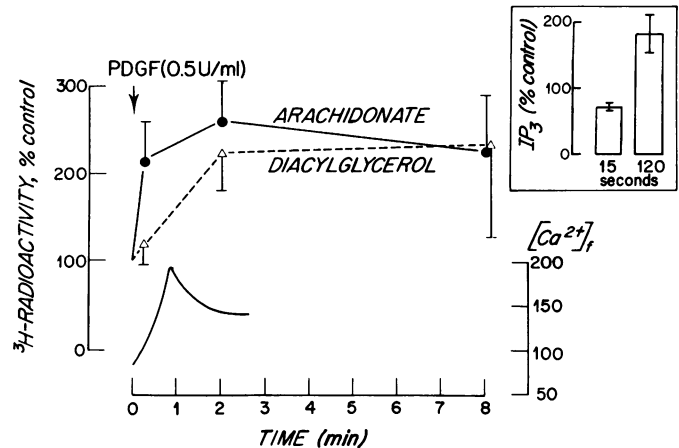


FIGURE 4. Time course of changes in cytosolic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ), free  $^3\text{H}$ -arachidonic acid,  $^3\text{H}$ -diacylglycerol, and inositol trisphosphate levels in mesangial cells after stimulation with platelet-derived growth factor (PDGF).  $[\text{Ca}^{2+}]_i$  was measured using the fluorescent probe, Fura-2. Data redrawn from Bonventre et al. (39). Figure taken from Bonventre (40). Reprinted with permission from Raven Press.

within the first 15 sec of PDGF stimulation. This indicates, therefore, that in response to PDGF there is a rapid increase in phospholipase  $\text{A}_2$  that precedes the peak in  $\text{Ca}^{2+}$  response. This suggests a  $\text{Ca}^{2+}$ -independent phospholipase C-independent mechanism for activation of phospholipase  $\text{A}_2$ . This phospholipase C-independent pathway for phospholipase  $\text{A}_2$  activation may involve GTP-binding proteins or may involve other events within the cell. For example, it is possible that the  $\text{Na}^+/\text{H}^+$  exchange activation that is seen by some investigators with PDGF in other cell types (41) may activate phospholipase  $\text{A}_2$  in the mesangial cell in a manner that is similar to that postulated in the platelet (42). Another possible explanation for the activation of phospholipase  $\text{A}_2$  might be a change in membrane potential which has been shown to activate the enzyme when incorporated into lipid bilayers (43).

### Synergistic Interactions with Calcium May Mediate Tissue Damage

The effects described above may help to explain how modest levels of increases in  $[\text{Ca}^{2+}]_i$  can mediate tissue damage by activation of phospholipase  $\text{A}_2$ , even though the calcium sensitivity of this enzyme would, at first glance, argue against the  $\text{Ca}^{2+}$ -mediated activation. An example of a  $\text{Ca}^{2+}$ -mediated detrimental action on a cell that is mediated by a synergistic interaction with another type of agonist, in this case, oxygen free radicals, is the  $\text{Ca}^{2+}$ , oxygen free radical-induced synergistic damage seen in renal mitochondria that is, likely, secondary to the activation of phospholipase  $\text{A}_2$  (44). With a variety of forms of ischemic and toxic injury cellular accumulation of  $\text{Ca}^{2+}$  and generation of oxygen free radicals have been implicated as mediators of cellular damage (9,24).

Potential damaging effects of these agents on mitochondrial function may result in impaired ATPase synthesis and abnormal cellular bioenergetic processes, leading ultimately to cell death. In order to model *in vitro* conditions mimicking the mitochondrial environment *in vivo* resulting from exposure of the cell to toxic and ischemic influences, mitochondria were exposed to  $\text{Ca}^{2+}$  and reactive oxygen species generated by hypoxanthine and xanthine oxidase in the presence of iron.  $\text{Ca}^{2+}$  treatment of mitochondria alone had no detrimental effects on mitochondrial bioenergetics. By contrast,  $\text{Ca}^{2+}$  pretreatment followed by the exposure to reactive oxygen species resulted in complete uncoupling of oxidative phosphorylation. There was an associated defect in the electron transport chain when site I substrates were used (Fig. 5). In addition the site I enzyme, NADH CoQ reductase, was examined directly and found to be markedly inhibited in response to  $\text{Ca}^{2+}$  and reactive oxygen species. The  $\text{F}_1\text{F}_0$  ATPase and adenine nucleotide translocase activities were also markedly inhibited by  $\text{Ca}^{2+}$  and reactive oxygen species. Again, in each case the reactive oxygen species and  $\text{Ca}^{2+}$  acted

synergistically, while  $\text{Ca}^{2+}$  alone had no adverse effect on the membranes. Also, in all cases dibucaine, an inhibitor of phospholipase  $\text{A}_2$ , protected the mitochondria either partially or completely against the adverse effects of  $\text{Ca}^{2+}$  and reactive oxygen species.

We have postulated one possible model for this synergistic interaction between  $\text{Ca}^{2+}$  and phospholipase  $\text{A}_2$  (44). Reactive oxygen species may initially increase the permeability of the mitochondria outer and inner membranes. Phospholipase  $\text{A}_2$ , located predominantly in the outer membrane, may be activated by mitochondrial  $\text{Ca}^{2+}$  influx. Lipid peroxidation by reactive oxygen species may enhance the susceptibility of these membranes to the action of activated phospholipase  $\text{A}_2$  (45). Phospholipase  $\text{A}_2$  may permeate the inner membrane gaining access to and causing degradation of the electron transport chain, ATPase, and translocase. The mitochondrial membrane has a high content of diphosphatidylglycerol (cardiolipin), a critical component of the catalytic subunits of the electron transport chain (17), ATP synthase (18,19) and adenine nucleotide translocase (20). With its high concentration of unsaturated fatty acids (46) and its

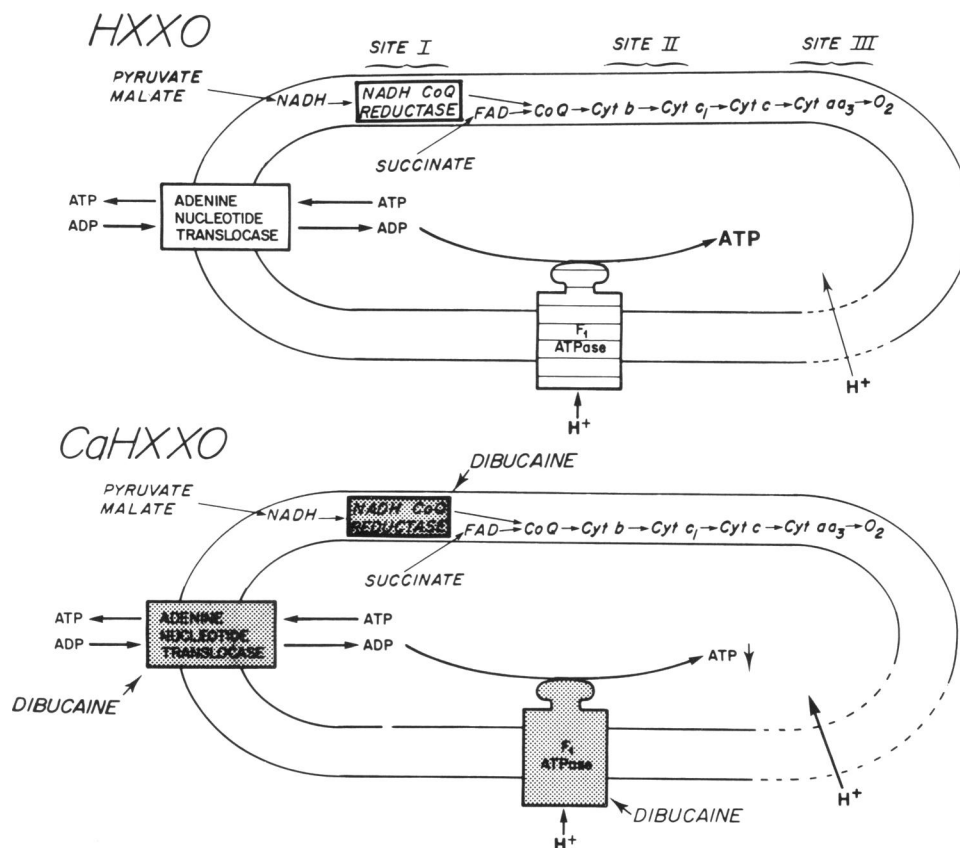


FIGURE 5. Effects of hypoxanthine (HX), xanthine oxidase (XO), and Fe in the absence (HXXO) or presence of  $\text{Ca}^{2+}$  (30 nmole/mg mitochondrial protein, CaHXXO) on renal cortical mitochondrial function. Calcium alone in these amounts had no effect upon mitochondrial function. Hypoxanthine and xanthine oxidase, in the presence of iron, increased mitochondrial membrane  $\text{H}^+$  permeability to a modest degree and also moderately impaired  $\text{F}_1\text{F}_0$  ATPase activity. By contrast, when the mitochondria took up an amount of  $\text{Ca}^{2+}$  that, of itself, had no adverse effects upon mitochondrial function, the subsequent exposure to hypoxanthine and xanthine oxidase resulted in a marked increase in inner membrane permeability, and impairment in electron transport chain site I (NADH CoQ reductase) enzymatic activity, adenine nucleotide translocase, and  $\text{F}_1\text{F}_0$  ATPase activities. Dibucaine mitigated the synergistic damaging effects of reactive oxygen species and  $\text{Ca}^{2+}$  upon the electron transport chain, adenine nucleotide translocase and  $\text{F}_1\text{F}_0$  ATPase. [Figure reproduced with permission from Thuren et al. (43)].



cone-shaped configuration (21), it may confer particularly high susceptibility of these mitochondrial sites to the action of phospholipase  $\text{A}_2$ .

## Summary

Fundamental to an understanding of the role of  $\text{Ca}^{2+}$  in physiological and pathophysiological processes, is the understanding of  $\text{Ca}^{2+}$  homeostasis and the documentation of changes in  $[\text{Ca}^{2+}]_i$  associated with various cell stimuli. However, in order to determine how  $[\text{Ca}^{2+}]_i$  is involved in a number of physiological and toxicological processes, it is important to take into account the complex interactions between  $\text{Ca}^{2+}$ , other second messengers, protein kinases and other inflammatory mediators. These multiple influences in the cell may work together synergistically to activate processes that mediate the physiological function of the cell agonist or the damaging effects on intracellular organelles or membranes. An important cellular enzyme, such as phospholipase  $\text{A}_2$ , which mediates a variety of normal and abnormal cellular responses, is not regulated simply by changes in levels of cytosolic free  $[\text{Ca}^{2+}]$  alone. The regulation of this and, probably, other critical cellular effector enzymes is much more intricate and, at the present time, incompletely characterized.

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## REFERENCES

- Bonventre, J. V., and Cheung, J. Y. Cytosolic free calcium concentration in cultured renal epithelial cells. *Am. J. Physiol.* 250: F329-F338 (1986).
- Bonventre, J. V., Skorecki, K. L., Kreisberg, J. I., and Cheung, J. Y. Vasopressin increases cytosolic free calcium concentration in glomerular mesangial cells. *Am. J. Physiol.* 251: F94-F102 (1986).
- Cheung, J. Y., Constantine, J. M., and Bonventre, J. V. Regulation of cytosolic free calcium concentration in cultured renal epithelial cells. *Am. J. Physiol.* 251: F690-F701 (1986).
- Krause, K. H., and Lew, P. D. Subcellular distribution of  $\text{Ca}^{2+}$  pumping sites in human neutrophils. *J. Clin. Invest.* 80: 107-116 (1987).
- Waite, M. *The Phospholipases*. Plenum Press, New York, 1987.
- Johnson, H. M., Russell, J. K., and Torres, B. A. Second messenger role of arachidonic acid and its metabolites in interferon- $\gamma$  production. *J. Immunol.* 137: 3053-3056 (1987).
- Dunn, M. J., Patrono, C., and Conotti, G. A. Prostaglandins and the Kidney. Plenum Medical Book Company, New York, 1983.
- Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R., and Lefkowitz, J. B. Arachidonic acid metabolism. *Ann. Rev. Biochem.* 55: 69-102 (1986).
- Bonventre, J. V., Leaf, A., and Malis, C. D. Nature of the cellular insult in ischemic acute renal failure. In: *Acute Renal Failure* (B. M. Brenner and J. M. Lazarus, Eds.), Churchill Livingstone, New York, 1988, pp. 3-43.
- Williams, T. J., and Morley, J. Prostaglandins as potentiators of increased vascular permeability in inflammation. *Nature* 246: 215-217 (1973).
- Chan, S. H., and Higgins, E., Jr. Uncoupling activity of endogenous free fatty acids in rat liver mitochondria. *Can. J. Biochem.* 58: 111-116 (1978).
- Chua, B. H., and Shrago, E. Reversible inhibition of adenine nucleotide translocation by long chain acyl-CoA esters in bovine heart mitochondria and inverted submitochondria particles. *J. Biol. Chem.* 252: 6711-6714 (1977).
- Lamers, J. M. J., and Hulsman, W. C. Inhibition of  $(\text{Na}^+ \text{K}^+)$  stimulated ATPase of heart by fatty acids. *J. Mol. Cell Cardiol.* 9: 343-346 (1977).
- Pitts, B. J. R., Tate, C. A., VanWinkle, W. B., Wood, J. M., and Entman, M. L. Palmitylcarnitine inhibition of the calcium pump in cardiac sarcoplasmic reticulum: a possible role in myocardial ischemia. *Life Sci.* 23: 391-401 (1978).
- Stenson, W. F., and Parker, C. W. Metabolism of arachidonic acid in ionophore-stimulated neutrophils. *J. Clin. Invest.* 64: 1457-1465 (1979).
- Corr, P. B., Gross, R. W., and Sobel, B. E. Amphipathic metabolites and membrane dysfunction in ischemic myocardium. *Circ. Res.* 55: 135-154 (1984).
- Fry, M., and Green, D. E. Cardiolipin requirement for electron transfer in complex I and III of the mitochondrial respiratory chain. *J. Biol. Chem.* 256: 1874-1880 (1981).
- Agarwal, N., and Kalra, V. K. Purification and functional properties of the DCCD-reactive proteolipid subunit of the  $\text{H}^+$  translocating ATPase from *Mycobacterium phlei*. *Biochem. Biophys. Acta* 723: 150-159 (1983).
- Santiago, E., Lopez-Moratalla, N., and Segovia, J. F. Correlation between losses of mitochondrial ATPase activity and cardiolipin degradation. *Biochem. Biophys. Res. Commun.* 53: 439-445 (1973).
- Beyer, K., and Klingenberg, M. ADP/ATP carrier protein from beef heart mitochondria has high amounts of tightly bound cardiolipin, as revealed by  $^{31}\text{P}$  nuclear magnetic resonance. *Biochemistry* 24: 3821-3826 (1985).
- Buckley, J. T. Cone-shaped lipids increase the susceptibility of phospholipids in bilayers to the action of phospholipases. *Can. J. Biochem. Cell Biol.* 63: 263-267 (1985).
- Bonventre, J. V., and Swidler, M. Calcium dependency of prostaglandin  $\text{E}_2$  production in rat glomerular mesangial cells. Evidence that protein kinase C modulates the  $\text{Ca}^{2+}$ -dependent activation of phospholipase  $\text{A}_2$ . *J. Clin. Invest.* 82: 168-176 (1988).
- Frei, E., and Zahler, P. Phospholipase  $\text{A}_2$  from sheep erythrocyte membranes.  $\text{Ca}^{2+}$  dependence and localization. *Biochem. Biophys. Acta* 550: 450-463 (1979).
- Bonventre, J. V. Mediators of ischemia renal injury. *Ann. Rev. Med.* 39: 531-544 (1988).
- Cheung, J. Y., Leaf, A., and Bonventre, J. V. Mitochondrial function and intracellular calcium in anoxic cardiac myocytes. *Am. J. Physiol.* 250: C18-C25 (1986).
- Ballou, L. R., and Cheung, W. Y. Marked increase of human platelet phospholipase  $\text{A}_2$  activity *in vitro* and demonstration of an endogenous inhibitor. *Proc. Natl. Acad. Sci. (U.S.)* 80: 5203-5207 (1983).
- Gietzen, K. Comparison of the calmodulin antagonists Compound 48/80 and calmidazolium. *Biochem. J.* 216: 611-616 (1983).
- Hidaka, H., Sasaki, Y., Tanaka, T., Endo, T., Ohno, S., Fujii, Y., and Nagata, Y. N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide, a calmodulin antagonist, inhibits cell proliferation. *Proc. Natl. Acad. Sci. (U.S.)* 78: 4354-4357 (1981).
- Hirata, F., Notsu, Y., Yamada, R., Ishihara, Y., Wano, Y., Kunos, I., and Kunos, G. Isolation and characterization of lipocortin (lipomodulin). *Agents and Actions* 17: 263-266 (1985).
- Davidson, F. F., Dennis, E. A., Powell, M., and Glenney, J. R., Jr. Inhibition of phospholipase  $\text{A}_2$  by lipocortins and calpactins. *J. Biol. Chem.* 262: 1698-1705 (1987).
- Jelsema, C. L. Light activation of phospholipase  $\text{A}_2$  in rod outer segments of bovine retina and its modulation by GTP-binding proteins. *J. Biol. Chem.* 262: 163-168 (1987).



32. Burch, R. M., Luini, A., and Axelrod, J. Phospholipase A<sub>2</sub> and phospholipase C are activated by distinct GTP-binding proteins in response to  $\alpha_1$ -adrenergic stimulation in FRTL5 thyroid cells. *Proc. Natl. Acad. Sci. (U.S.)* 83: 7201-7205 (1986).
33. Burch, R. M., and Axelrod, J. A GTP-binding protein (G-protein) regulates phospholipase A<sub>2</sub> in RAW264.7 macrophages. *Fed. Proc.* 46: 703 (1987).
34. Schlondorff, D., Satriano, J. A., and DeCandido, S. Different concentrations of pertussis toxin opposite effects on agonist-induced PGE<sub>2</sub> formation in mesangial cells. *Biochem. Biophys. Res. Commun.* 141: 39-45 (1986).
35. Pfeilschifter, J. and Bauer, C. Pertussis toxin abolishes angiotensin II-induced phosphoinositide hydrolysis and prostaglandin synthesis in rat renal mesangial cells. *Biochem. J.* 236: 289-294 (1986).
36. Moolenaar, W. H., Tertoolen, L. G. J., and de Laat, S. W. Phorbol ester and diacylglycerol mimic growth factors in raising cytosolic pH. *Nature* 312: 371-374 (1984).
37. Cantiello, H. F., Angel, J. B., Ausiello, D. A., and Bonventre, J. V. Ca modulates a Na/H exchanger in mesangial cells. *Kidney Int.* 31: 162 (1987).
38. Sweatt, J. D., Connolly, T. M., and Cragoe, E. J., and Limbird, L. E. Evidence that Na<sup>+</sup>/H<sup>+</sup> exchange regulates receptor-mediated phospholipase A<sub>2</sub> activation in human platelets. *J. Biol. Chem.* 261: 8667-8673 (1986).
39. Bonventre, J. V., Weber, P. C., and Gronich, J. H. PAF and PDGF increase cytosolic [Ca<sup>2+</sup>] and phospholipase activity in mesangial cells. *Am. J. Physiol.* 254: F87-F94 (1988).
40. Bonventre, J. V. Vasopressin and calcium in cultured glomerular mesangial cells. In: *Vasopressin: Cellular and Integrative Functions* (A. W. Cowley, Jr., J.-F. Liard, and D. A. Ausiello, Eds.), Raven Press, New York, 1988, pp. 89-96.
41. Moolenaar, W. H. Effects of growth factors on intracellular pH regulation. *Ann. Rev. Physiol.* 48: 363-376 (1986).
42. Sweatt, J. D., Connolly, T. M., Cragoe, E. J., and Limbird, L. E. Evidence that Na<sup>+</sup>/H<sup>+</sup> exchange regulates receptor-mediated phospholipase A<sub>2</sub> activation in human platelets. *J. Biol. Chem.* 261: 8667-8673 (1986).
43. Thuren, T., Tulkki, A.-P., Virtanen, J. A., and Kinnunen, P. K. J. Triggering of the activity of phospholipase A<sub>2</sub> by an electric field. *Biochemistry* 26: 4907-4910 (1987).
44. Malis, C. D., and Bonventre, J. V. Mechanism of calcium potentiation of oxygen free radical injury to renal mitochondria. A model for post-ischemic and toxic mitochondrial damage. *J. Biol. Chem.* 261: 4201-4208 (1986).
45. Sevanian, A., and Kim E. Phospholipase A<sub>2</sub> dependent release of fatty acids from peroxidized membranes. *J. Free Radicals Biol. Med.* 1: 263-271 (1985).
46. Gray, G. M. The isolation of phosphatidylglycerol from rat liver mitochondria. *Biochem. Biophys. Acta* 84: 35-40 (1984).